Loricrin Expression Is Coordinated with Other Epidermal Proteins and the Appearance of Lipid Lamellar Granules in Development

Jackie R. Bickenbach, Jeanette M. Greer, Donnie S. Bundman, Joseph A. Rothnagel, and Dennis R. Roop
Departments of Cell Biology and Dermatology, Baylor College of Medicine, Houston, Texas, U.S.A.

In mouse, epidermal development proceeds from a single basal cell layer covered by a specialized single cell layer called the periderm at E14 to a fully differentiated stratified squamous epithelium at E18. To determine when loricrin, a major cell envelope component, is expressed during development, we examined fetal skin from mice of gestational ages E13 through E19 and compared the temporal pattern of loricrin expression with that of other differentiation markers. We found that loricrin mRNA and protein were expressed by E16, following the expression of keratins K1 and K10 and preceding the expression of profilaggrin. Interestingly, both loricrin and profilaggrin were initially expressed focally in areas corresponding to more advanced morphologic stages of maturation. Because the cornified envelope is a composite structure consisting of both protein and lipid components, we also monitored the appearance of lipid lamellar granules during epidermal development. These granules were first evident at E16 and the extrusion of lipids from the granules into the intercellular space occurred at E17, prior to the cross linking of loricrin into the cell envelope. Our results document that loricrin is expressed and accumulates at the cell periphery subsequent to the extrusion of lipids, but prior to processing of profilaggrin. We suggest that the sequential regulation of these events is critical for formation of epidermal barrier function during development. *Key words: cell envelope/skin/keratin. J Invest Dermatol 104: 405–410, 1995*

In mouse, gestational development of a complete stratified squamous epidermis takes place in about 4 d, from E15 to E18 [1]. During this time, keratinocytes begin to produce the specific differentiation markers that are found in newborn epidermis. The differentiation-specific keratins (K1 and K10) are present as soon as the epidermis forms its intermediate second cell layer, which corresponds to the adult spinous layer [2–6], and histidine-rich profilaggrin appears with the formation of the granular layer [7–9]. In the final stage of maturation of the developing epidermis, a highly insoluble cornified cell envelope is assembled just beneath the plasma membrane [10] concomitant with the condensation of keratin filaments to form the lifeless squames of the stratum corneum. This cornified envelope is comprised of several proteins crosslinked into a rigid scaffold [11] with specialized lipids covalently attached to the intercellular surface [12].

In adult epidermis, several proteins have been identified as potential precursors of the cornified envelope [11]. Of these proteins, loricrin is the most abundant component found in the cell envelope [11,13,14]. Previous studies employing immunoelectron microscopy (IEM) initially detected loricrin in discrete granules in the granular layer of the epidermis and subsequently at the periphery of cells throughout the stratum corneum [15]. IEM labeling of purified cell envelopes established that loricrin was only detectable on the inner surface [13,15]. On the basis of these results, it was concluded that loricrin was incorporated into the cell envelope as a very late event. Because other cell envelope components, such as involucrin [16], are expressed at an earlier stage of differentiation than loricrin, and the staining pattern for involucrin becomes weaker in the transition from the granular layer to stratum corneum where it is essentially absent [16], it is likely that these cell envelope precursors are cross-linked to form a scaffold upon which loricrin is later incorporated [17,18]. Given the location of loricrin within the cell envelope and its unusual protein structure, i.e., repeating arrays of glycine-serine-rich peptides interspersed with tyrosine and cysteine residues [13,14] that are predicted to form secondary structures similar to Ω loops [14], it has been proposed that loricrin may act as an attachment site for the filament/matrix complex, via similar Ω loop structures in the end domains of keratins 1 and 10 [19].

In a previous study we showed that loricrin transcription was confined to the upper spinous and granular layers of newborn mouse epidermis [13] and followed that of K1 and K10, occurring at approximately the same stage of differentiation as filaggrin [13,20]. However, a detailed examination of loricrin expression during epidermal development has not been reported. To determine the exact timing of loricrin expression during epidermal development, we examined murine fetal skin for both mRNA and protein expression of K1, K10, K14, filaggrin, and loricrin. In addition, using antibodies specific for each protein, we compared the morphological expression and localization of each during development. We also used electron microscopy to follow the

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Reprint requests to: Dr. Dennis R. Roop, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

Abbreviation: E13-E19, embryonic days 13 through 19 of gestation.

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appearance of loricrin, filaggrin, and lipid lamellar granules and correlated the timing of extrusion of lipids into the intercellular space with formation of the cell envelope.

MATERIALS AND METHODS

Dissection of Skin Samples Male and female ICR mice (Harlan Sprague-Dawley, Houston, TX) were mated at 5:00 PM and the males removed 14 h later. We estimated our gestational times, following the convention established in previous reports on mouse skin development [1], and compared relative times of expression of protein and mRNA within each group. On days 13 through 19 we sacrificed pregnant female mice and removed the embryos. The back skin from at least seven embryos was examined for each time period. To ensure that the same gestation time was analyzed for protein and mRNA, back skin from each embryo was dissected away from the underlying muscle and divided longitudinally into strips from shoulder to rump. Skin was frozen in OCT for immunofluorescence, stored dried at −20°C for Western analysis, stored in liquid nitrogen for RNA analysis, and processed for electron microscopy.

Isolation of Proteins and Analysis by Western Immunoblots Epidermal proteins were extracted from the back skin of embryos from each time period, using 20% 2-mercaptoethanol and 5% sodium dodecylsulfate as previously described [21], then separated by gel electrophoresis [22], blotted onto nitrocellulose [23], and detected by an immuno-enzymatic reaction as previously described using rabbit anti-K1, K10, or K14 [24,25], rabbit anti-filaggrin [13], or rabbit anti-loricrin [14]. The immunoblot for each protein examined was made from the same gel, which had equal amounts of protein loaded for each time period. As we previously reported [21], standard protein quantitation assays cannot be used when epidermal particulate proteins are isolated; therefore, proteins were loaded equally according to density of coomasie blue staining.

Detection of RNA by RT-PCR Skin from all embryos from each time period was ground to a powder in liquid nitrogen. Total RNA was extracted with RNAzol (Cinna/Biotecx Labs, Inc., Houston, TX), solubilized with chloroform, and precipitated with 2-propanol. The pellets were air dried, resuspended in 1 mM ethylenediaminetetraacetic acid at a concentration of 1 μg/μl as determined by optical density 260 nm, and stored at −80°C until ready for the polymerase chain reaction (PCR). The same sample from each time point was used for each PCR. Random primed cDNA was synthesized from equal amounts of RNA by using the Superscript Reverse Transcriptase Kit from GIBCO-BRL (Gaithersburg, MD). The cDNAs were then amplified by PCR with specific oligos for MK1 (5′-ATTTGCCAGAGGAGCAAGGC-3′, 5′-TGGGAGTGCACTCTCCAGAC-3′), MK10 (5′-GGCCAGCTACTCGGACAAAGTCCGGGC-3′, 5′-AAGGGTCAGCTCATCCAG-3′), filaggrin (5′-GCTTAAATGCATCTCCAG-3′, 5′-AGTCAGTCCTATTGCAGG-3′), and loricrin (5′-GGTTCCCCTTCTCCTTAAAC-3′, 5′-CTCCACCAGAGGTCTTTCC-3′), designed to yield a product size of 417 bp for MK1, 350 bp for MK10, 330 bp for filaggrin, and 100 bp for loricrin. Primers were chosen to amplify across an intron boundary to ensure amplification of mRNA. Because loricrin was difficult to amplify by PCR, due to the unusually high G-C content of the coding region (73%) [13], we selected primers that would only amplify a short 5′ noncoding sequence. The variable level of signals obtained by RT-PCR is most likely due to the inherent characteristics of the methodology.

The 100 μl of PCR reaction contained 200 ng of each primer (except MK10 contained 600 ng of each primer), 10 μl of 10 × buffer (Promega), 2 μl of 10 mM dNTP, 100 μg of DNA, and 1 μl of Taq DNA polymerase. Samples were heated to 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min each, annealing at 50°C for 1 min, and extension at 72°C for 1 min; the final extension was at 72°C for 15 min. PCR products were analyzed by 2% Metaphor (FMC BioProducts) gel electrophoresis.

Detection of Proteins by Immunofluorescent Antibodies A three-step, two-color indirect immunofluorescence was performed by sequential incubation of frozen sections with primary polyclonal antibodies (rabbit anti-K1, K10, K13, filaggrin, or loricrin, and guinea pig anti-K14); secondary antibodies (sheep anti-rabbit conjugated with FITC [Dakopatts, Denmark], and goat anti-guinea pig conjugated with biotin [Vector, Burlingame, CA]); and streptavidin Texas red (GIBCO-BRL, Gaithersburg, MD). This procedure has been described in detail previously [26].

Identification of Loricrin and Lipids by Electron Microscopy Techniques Pieces of back skin from developing mouse embryos were processed for electron microscopy by three different methods: the standard method allowed general examination of the tissue and comparison to adult tissue processed in the same manner; the IEM method allowed antibody reactions for the various proteins; and the lipid method allowed visualization of the lipid lamellar granules and the extruded lipid lamellae that are not seen by ordinary processing methods.

The Standard Method: Tissue pieces were fixed in half-strength Karnovsky's fixative at 4°C [27], post-fixed in osmium tetroxide, *en bloc* stained with uranyl acetate, dehydrated through a graded series of ethanol into propylene oxide, and embedded in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA). The subsequent blocks were cut at 60 nm and the sections stained with uranyl acetate and lead citrate.

The IEM Method: Tissue pieces were fixed 2 h in half-strength Karnovsky's fixative [27]; washed in 0.1 M cacodylate buffer, pH 7.0, at room temperature; dehydrated through a graded series of room-temperature ethanol; infiltrated in Unicryl Resin (Goldmark Biologicals, Phillipsburg, NJ) overnight at room temperature, and embedded in Unicryl; and the blocks were polymerized with 360 nm ultraviolet light. The blocks were cut at 80 nm and the sections stained overnight in rabbit anti-loricrin antibody, diluted 1/100 in Tris/bovine serum albumin buffer, then 1 h in 1/10 dilution of goat anti-rabbit antibody conjugated with 30-nm gold particles (Goldmark Biologicals), and post-stained in uranyl acetate and lead citrate.

The Lipid Method: Tissue pieces were fixed overnight in half-strength Karnovsky's fixative [27] at 4°C, rinsed in 0.1 M cacodylate buffer, fixed for 30 min at room temperature in 1% osmium tetroxide and 1.5% potassium ferrocyanide, rinsed in 0.1 M cacodylate buffer, dehydrated through a graded series of ethanol, and embedded in EMbed 812 (Electron Microscopy Sciences). The subsequent blocks were cut at 60 nm and the sections stained with uranyl acetate and lead citrate.

RESULTS

Expression of the major differentiation markers in epidermis was ascertained using fetal murine skin from timed matings **(Figs 1–3)**. The expression of the individual proteins is clearly shown by immunoblot analysis **(Fig 1)**. K14 was already expressed by E13, K1, and K10 from E15, loricrin from E16, and filaggrin from E17. It is important to note that filaggrin was initially detected in the high–molecular-weight profilaggrin form at E17, and only detected as the low–molecular-weight processed form 24 h later, suggesting that filaggrin is not fully processed until E18. In adult, filaggrin is initially synthesized in granular cells as a large polyprotein precursor, profilaggrin [28], that is subjected to a number of post-translation modifications to liberate the smaller filaggrin molecules [29].

The RT-PCR data **(Fig 2)** show that the mRNA for K1 was induced by E14, prior to K10 at E15. The appearance of both mRNAs preceded their respective proteins, and both continued to be expressed through E19. Loricrin expression was detected by E16 and that for profilaggrin by E17. Both granular layer markers continued to be expressed through E19.

Immunofluorescent analysis demonstrated that K14 was only detected in the basal cell layer at E14 when the developing epidermis consisted of this cell layer and a periderm layer **(Fig 3)**. Our previous analysis of neonatal epidermis has shown that K14 is expressed in basal cells and is transcriptionally down-regulated once cells commit to terminal differentiation [30]. However, K14 staining persists into the upper cell layers of the epidermis because of the inherent stability of keratin proteins. Thus, in developing epidermis K14 continued to be detected throughout the nucleated epidermal layers at all subsequent time periods examined (red and yellow staining in **Fig 3**, although staining was diminished in the granular layer. The differentiation-specific keratin proteins, K1 and K10, which are expressed in adult epidermal spinous cells, were not detected until E15 (yellow staining in **Fig 3*c,d***), when the developing epidermis consisted of two cell layers, and persisted in the suprabasal nucleated cells throughout the time course examined (yellow or green staining in **Fig 3*c,d,e,f,h,i,l,m***). The apparent lack of K1 staining in the stratum corneum **(Fig 3*l*)** is believed to be due to proteolytic processing of this protein, resulting in the removal of the epitope recognized by our antibody [24]. The late differentiation marker filaggrin, which is expressed in adult granular cells, was initially detected at E17 as a punctate green staining in a few granular cells (granular green staining in cell shown **Fig 3*k***), but by

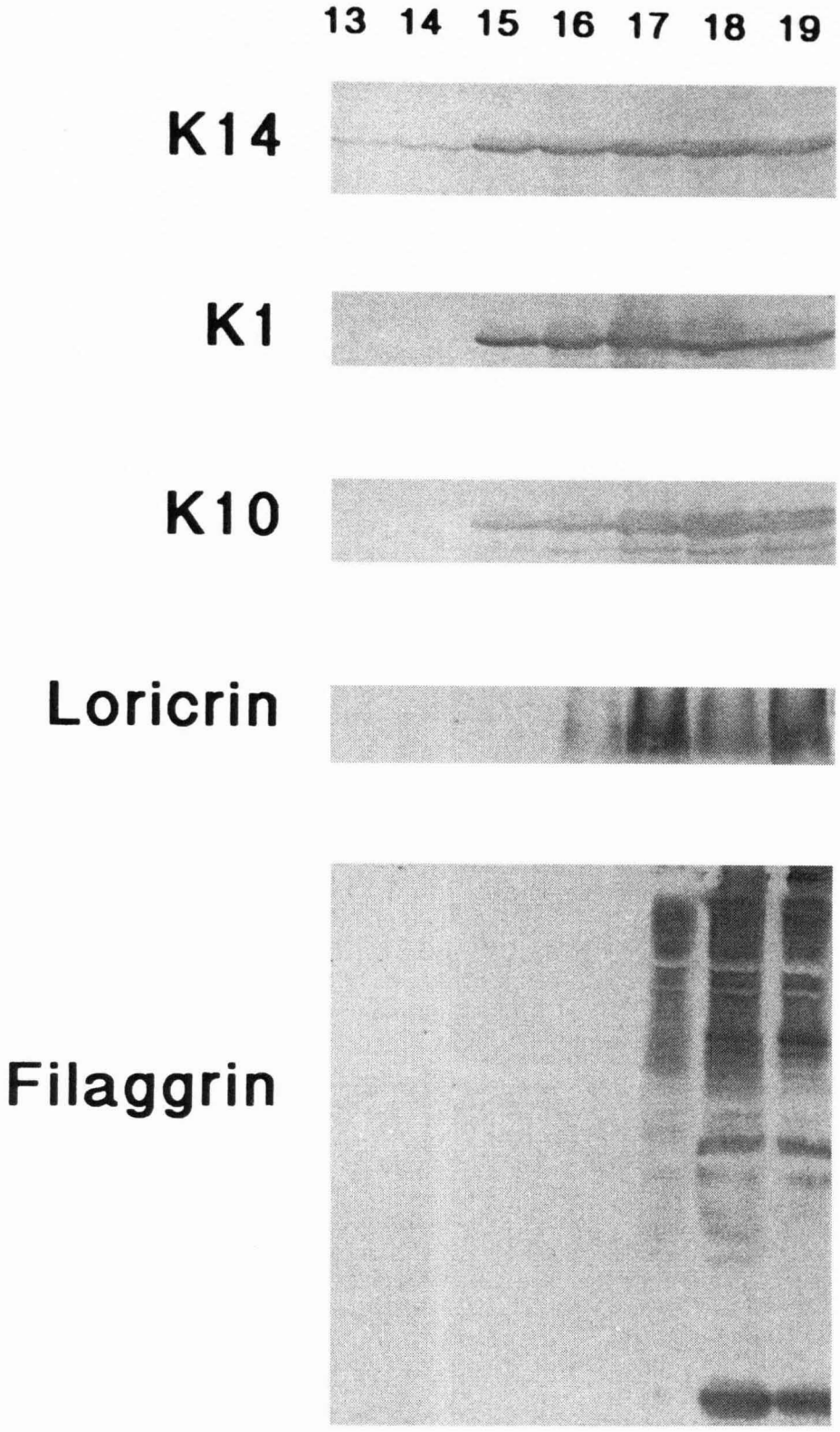

Figure 1. Immunoblot analysis of epidermal proteins in developing mouse epidermis. Gestational ages of E13–E19 are represented. Equal amounts of protein, determined by density of coomasie blue staining [21], were loaded for each time period, and the blot for each marker was made from the same gel.

Figure 2. Reverse transcriptase PCR analyses showing expression of mRNA in developing mouse epidermis. Gestational ages E14–E19, respectively; M, molecular weight markers. One hundred nanograms of cDNA was used in each respective PCR reaction, and 5 μl of resulting PCR product was loaded on the gel.

E18 it was observed in all cells in the granular layer (yellow and green staining in **Fig 3*o***).

Immunofluorescent analysis shows that the onset of loricrin expression during development does not occur in a uniform manner. Staining was patchy and somewhat particulate in some, but not all cells of the uppermost layer of E16 epidermis (see green staining in **Fig 1*g*** for an example of a stained cell). This corresponds to the less intense signal for loricrin at E16 seen in the immunoblot of **Fig 1**. IEM determined that loricrin was deposited in amorphous granules in the uppermost cell layer of E16 epidermis **(Fig 4*a,b*)**, resembling those previously reported for newborn mouse epidermis [15]. By E17, loricrin was readily detectable and showed intense staining at the peripheries of all cells in the granular and stratum corneum layers (green staining in **Fig 3*j,n***). In addition, loricrin was not detected in the periderm at any time period [see **Fig 3*p*** where the periderm from E18 epidermis is stained with antibody to K13 (red stain), but not with antibody to loricrin (green stain); note that the periderm was beginning to detach from the underlying epidermis and in this section it was partly folded over the epidermis].

Lipid lamellar granules were first detected at E16 in the epidermal cell layer just beneath the periderm **(Fig 4*c*)**, concomitant with the appearance of loricrin granules. These lipid granules, also found in the granular cells in adult epidermis, are easily identified by their characteristic lamellae [31]. In adult epidermis, lipid lamellae are extruded between the uppermost granular cells and the stratum corneum [31]. In developing mouse epidermis, this extrusion occurred by E17, when lipid lamellae were detected between the uppermost epidermal cell and the periderm **(Fig 4*d*)**. This occurred prior to the cross linking of loricrin into the cell envelope, as shown by the lack of any thickening of the epidermal cell membrane in **Fig 4*d***.

DISCUSSION

We have determined the timing of loricrin expression during mouse development by observing the onset of expression of loricrin relative to that of other epidermal differentiation markers (see **Table I**). The temporal pattern of expression observed for K14, K1, K10, and filaggrin in developing mouse epidermis in this study corresponds to what has been previously reported for human development [2,7] and for other mammalian species [3–6,8,9]. Two preliminary studies on loricrin expression in developing

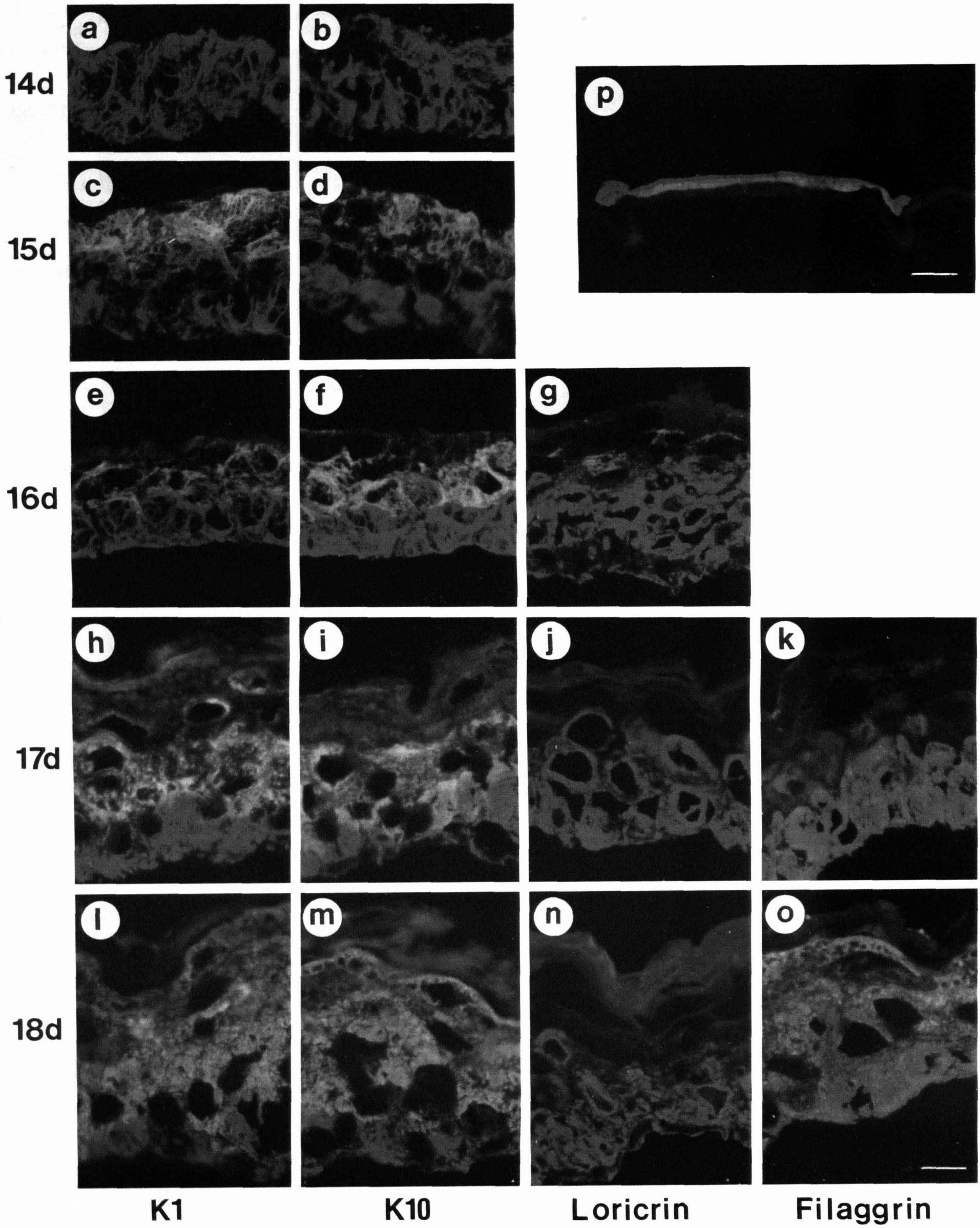

Figure 3. Double-label immunofluorescence analysis of developing mouse epidermis. Antibody to K14 (labeled with Texas Red) was used as a counterstain in all micrographs, except *p*. Note that K14 stains only the epidermal cells and not the periderm; thus K14 stains only one cell layer at E14, two at E15, three at E16, and four at E17 and E18, and that the intensity of stain is strongest in the basal layer and diminishes toward the granular layer. Fluorescein isothiocyanate–labeled antibodies reacted with E14 epidermis for K1 (*a*) and K10 (*b*); E15 for K1 (*c*) and K10 (*d*); E16 for K1 (*e*), K10 (*f*), and loricrin (*g*); E17 for K1 (*h*), K10 (*i*), loricrin (*j*), and filaggrin (*k*); and E18 for K1 (*l*), K10 (*m*), loricrin (*n*), and filaggrin (*o*) (*scale bar,* 15 μ). Insert (*p*) shows E18 epidermis reacted with Texas Red–labeled antibody to K13, which stains the periderm, and fluorescein isothiocyanate–labeled antibody to loricrin, which stains the upper layers of the epidermis, but not the periderm (*scale bar,* 60 μ). Areas containing epitopes recognized by both K14 and the other antibodies stain yellow or orange.

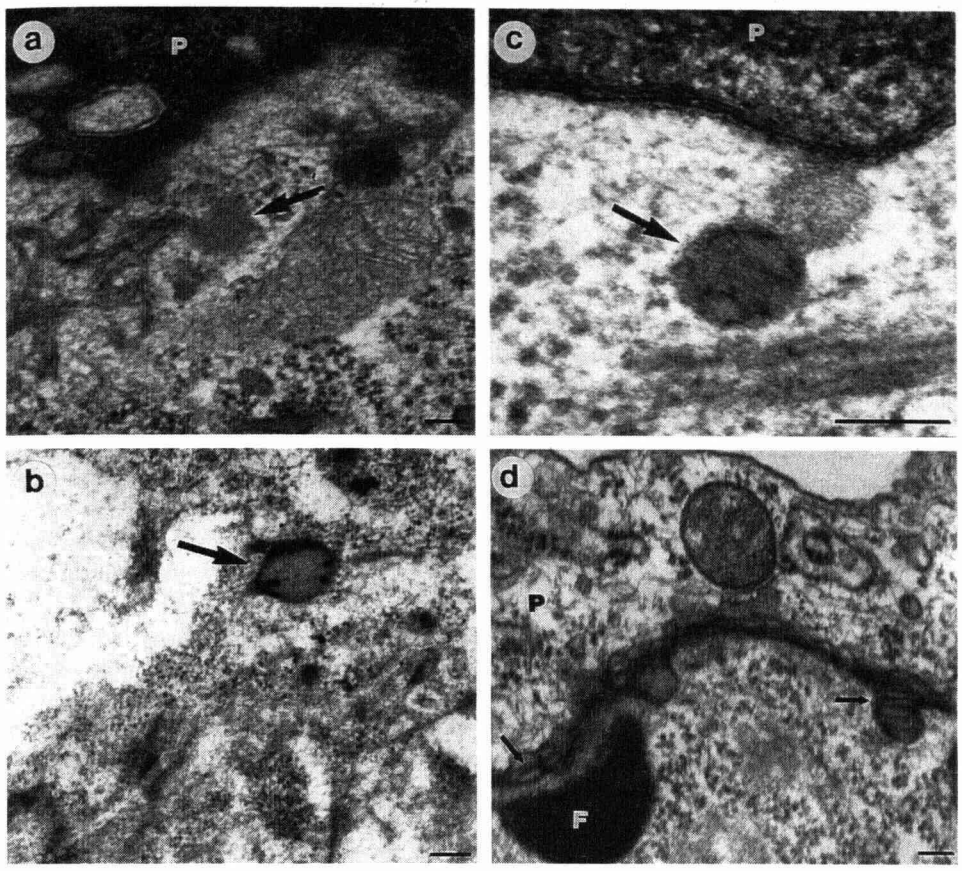

Figure 4. Electron microscopic analysis of loricrin, filaggrin, and lipid lamellar granules in developing mouse epidermis. *a*) Loricrin granule (*arrow*) in uppermost cell of E16 epidermis. *b*) Loricrin granule in E16 epidermis stained with antibody to loricrin and visualized with secondary antibody conjugated with 30-nm gold particles. *c*) Lipid lamellar granule (*arrow*) in uppermost cell of E16 epidermis. *d*) Filaggrin granule (F) present in uppermost cell of E17 epidermis and lipid lamellae (*arrows*) extruded between the uppermost epidermal cell layer and the periderm; note the absence of a cell envelope. (*Scale bars,* 100 nm; P, periderm.)

epidermis have been reported: Holbrook, *et al** reported that loricrin protein was expressed in the early intermediate cell layers of human fetal epidermis at 7 to 8 weeks gestation, and Yoneda and Steinert [32], examining the over-expression of human loricrin in transgenic mice by RT-PCR, briefly stated that endogenous mouse loricrin mRNA was expressed by E16, which agrees with the present study. In addition, we show that loricrin protein is first detected at E16, and its histologic localization with respect to the specific stages of mouse epidermal development demonstrates that loricrin is initially expressed focally with the protein appearing in only a few granular cells. We also show that loricrin is not present in the periderm. This finding is in agreement with the study on human fetal skin,* in which involucrin and keratolinin were present in the periderm as early as eight weeks gestation, but loricrin was not detected in the periderm at any age examined.

In mouse epidermis, the period between E16 and E17 is a crucial time in the development of barrier function because the periderm, the initial protective barrier of developing epidermis, begins to detach from the underlying fetal epidermis at E18. At this time the fetal epidermis must be able to function as an effective protective barrier. Thus, the fetal granular and stratum corneum cells must form strong but flexible cell envelopes, strengthen the cytoskeletal network, and create an impermeable water barrier. The formation of a structurally mature cornified cell envelope occurs in several steps (see for review [17,18]). Initially, in response to an increase in calcium concentration, transglutaminase K cross-links a membrane-bound protein with a cytostolic precursor protein (most likely involucrin). This initial scaffold is further strengthened by cross-linking other less abundant components until the inner surface of the keratinocyte cell membrane is covered. This inner surface envelope is then reinforced by the attachment of loricrin by transglutaminase E. Keratin filaments, which have been aggregated by filaggrin, may then interact with the thickened protein envelope to strengthen the cytoskeleton. Finally, lipids are covalently bound to the outer surface of the protein cell envelope to form the complete water barrier. It is of interest that we first detect loricrin granules and lipid lamellar granules at the beginning of this crucial time (E16). In addition, loricrin is not cross-linked into the cell envelope until E17, just after extracellular lipid multilamellar structures are first detected between the uppermost epidermal cell layer and the periderm. Although the cross-linking of loricrin into the cell envelope seems to be well-timed to interact with the newly extruded lipids, it is unlikely that the extracellular lipids are covalently bound to loricrin, because it is located on the inside of the cell envelope [13,15]. Instead, it has been suggested that the lipids bind to a specific conformation of involucrin [33]. Perhaps the cross-linking of loricrin changes the conformation of the cell envelope such that the extracellular lipids can be covalently bound to the exterior surface, thus completing the barrier.

Our findings clearly document that K1, K10, loricrin, and filaggrin are expressed at precise times during development corresponding to distinct morphologic events. Additionally, we have determined that the synthesis of lipids and their extrusion into the intercellular space is tightly integrated into this coordinated program. These observations, coupled with the Western blot analysis indicating that processed filaggrin only becomes abundant at E18, suggest that the filament/matrix complex, primarily composed of K1 and K10 [29] in association with filaggrin, forms as a very late event in development. It has been postulated that the end domains of keratins 1 and 10 [19] may interact with the glycine-serine-rich loops of loricrin. Our data suggest that loricrin is fully cross-linked into the cell envelope and in position to provide these putative attachment sites for the filament/matrix complex at the time of its formation during development. The temporal sequence of events documented in this study is undoubtedly critical for formation of a functional epidermal barrier during development.

* Holbrook KA, Underwood RA, Dale BA, Thacher SM, Wuepper KD, Banks-Schlegel S: Cornified cell envelope (CCE) in human fetal skin: involucrin, keratolinin, loricrin, and transglutaminase expression and activity (abstr 64). *J Invest Dermatol* 96:542, 1991.

Table I. Timing of Differentiation Events in Developing Mouse Epidermis

	E13	E14	E15	E16	E17	E18	E19
Number of epidermal cell layers	1	1	2	3	4	5	6
Periderm	← tightly attached				loosely attached →		shedding
K14	| expressed at all time periods examined →						
K1	| →						
K10	| →						
Lipids				granules →	extruded →		
Loricrin				granules →	cross linked →		
Filaggrin					profilaggrin →	processed →	
Stratum Corneum						| →	

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